IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

LALVANI et al

Serial No. **09/308,725**

Filed: **May 24, 1999**

Title: ASSAY METHOD FOR PEPTIDE SPECIFIC T-CELLS

Atty Dkt. **117-310**
C# M#
Group Art Unit:
Examiner:
Date: **March 22, 2000**

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

SUBMISSION OF PRIORITY DOCUMENT

This is a response/amendment/letter in the above-identified application and includes an attachment which is hereby incorporated by reference and the signature below serves as the signature to the attachment in the absence of any other signature thereon.

Fees are attached as calculated below:

Total effective claims after amendment **0** minus highest number previously paid for **20** (at least 20) = 0 x $ 18.00			$ 0.00
Independent claims after amendment **0** minus highest number previously paid for **3** (at least 3) = 0 x $ 78.00			$ 0.00
If proper multiple dependent claims now added for first time, add $260.00 (ignore improper)			$ 0.00
Petition is hereby made to extend the current due date so as to cover the filing date of this paper and attachment(s) ($110.00/1 month; $380.00/2 months; $870.00/3 months)			$ 0.00
Terminal disclaimer enclosed, add $ 110.00			$ 0.00
First submission after Final Rejection pursuant to 37 CFR 1.129(a) ($690.00)			$ 0.00
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		Subtotal	$ **0.00**
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The Commissioner is hereby authorized to charge any deficiency in the fee(s) filed, or asserted to be filed, or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Account No. 14-1140. A duplicate copy of this sheet is attached.

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NIXON & VANDERHYE P.C.
By Atty: Mary J. Wilson, Reg. No. 32,955

Signature: Mary J. Wilson

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* * * * * * * * * * * *

March 22, 2000

Assistant Commissioner for Patents
Washington, DC 20231

SUBMISSION OF PRIORITY DOCUMENT

Sir:

It is respectfully requested that this application be given the benefit of the foreign filing date under the provisions of 35 U.S.C. §119 of the following, a certified copy of which is submitted herewith:

Application No.	Country of Origin	Filed
9624456.1	United Kingdom	25 November 1996

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: Mary J. Wilson

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The Patent Office

INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
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I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

I also certify that the attached copy of the request for grant of a Patent (Form 1/77) bears an amendment, effected by this office, following a request by the applicant and agreed to by the Comptroller-General.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

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Signed Andrew Gerrey

Dated 8 March 2000

Patents Form 1/77

Patents Act 1977
(Rule 16)

THE PATENT OFFICE
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1/77

26NOV96 E234555-1 D00085
P01/7700 25.00

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference — PP/1064

2. Patent application number *(The Patent Office will fill in this part)* — 25 NOV 1996 — **9624456.1**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

ISIS INNOVATION LIMITED
2 South Parks Road
OXFORD
OX1 3UB

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation — UNITED KINGDOM — 3998564001

4. Title of the invention — ASSAY METHOD

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

STEVENS HEWLETT & PERKIN
1 Serjeants' Inn
Fleet Street
LONDON
EC4Y 1LL

FS1/77
1/300CP

Patents ADP number *(if you know it)* — 1546003

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J A Kemp & Co
14 South Square
Gray's Inn
LONDON
WC1R 5LX

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

Yes

ASSAY METHOD

This invention is concerned with a method of assaying for activated peptide-specific T-cells. It is an adaptation of the known ELISPOT assay, which is reviewed in current protocols in Immunology, Unit 6.19, pages 6.19.1-8.

The filter immunoplaque assay, otherwise called the enzyme-linked immunospot assay (ELISPOT), was initially developed to detect and quantitate individual antibody-secreting B cells. At the time it was developed, the technique provided a rapid and versatile alternative to conventional plaque-forming cell assays. Recent modifications have improved the sensitivity of the ELISPOT assay such that cells producing as few as 100 molecules of specific protein per second can be detected. These assays take advantage of the relatively high concentration of a given protein (such as a cytokine) in the environment immediately surrounding the protein-secreting cell. These cell products are captured and detected using high-affinity antibodies.

The ELISPOT assay utilises two high-affinity cytokine-specific antibodies directed against different epitopes on the same cytokine molecule: either two monoclonal antibodies or a combination of one monoclonal antibody and one polyvalent antiserum. ELISPOT generates spots based on a colorimetric reaction that detects the cytokine secreted by a single cell. The spot represents a "footprint" of the original cytokine-producing cell. Spots are permanent and can be quantitated visually, microscopically, or electronically.

The ELISPOT assay involves five specific steps: (1) coating a purified cytokine-specific antibody to a nitrocellulose-backed microtitre plate; (2) blocking the plate to prevent nonspecific absorption of any other

1 CD8+ cell in 10^5 - 10^6 is a memory cell that has been pre-sensitised to a specific epitope associated with the influenza virus.

The method of the invention involves adding a peptide to the fluid. The peptide may be a known epitope for a well characterised viral infection; or may be a candidate epitope possibly associated with a less well characterised viral infection. The resulting fluid mixture is incubated under conditions to stimulate any peptide-specific T-cells that may have been pre-sensitised to that particular virus-derived peptide *in vivo*. The peptide needs to be of a length, e.g. 7 - 12 and particularly 8 - 10 amino acid residues long, that is recognised by CD8+ cells. It is supposed that the generality of the CD8+ cells (and other PBMC) present the peptide to the small minority of CD8+ cells that may have been pre-sensitised to the peptide. If such activated or pre-sensitised peptide-specific T-cells are present in the test fluid, they respond by secreting IFN-γ or other cytokine which then becomes bound to the immobilised antibody.

It is preferred that the peptide be added in uncombined form to the fresh cells. While it is possible to add cultured cells that have been pulsed with the peptide, this is not necessary when using defined peptide epitopes. The peptides should be added in an amount sufficient to generate an observable signal; a preferred concentration range in the fluid is 0.01 up to 100 μM particularly 0.5 - 5.0 μM.

Incubation should be continued for a time sufficient to permit CD8+ cells that have been pre-sensitised *in vivo* to the particular peptide chosen to secrete the IFN-γ or other cytokine. The incubation should not continue for so long that quiescent CD8+ cells have time to become activated by the peptide and start to secrete cytokines. This suggests an incubation time of 4 - 24 hours, more particularly 6 - 16 hours. It is an advantage of the invention that the incubation part of the test can be performed in a single working day or overnight, and without the use of sterile conditions required for cell culture *in vitro*.

b) It requires minimal technical equipment and is suitable for field conditions in the tropics and developing countries as well as routine diagnostic laboratories. The LDA, by contrast, requires many peripheral blood lymphocytes, a source of gamma irradiation to inactivate the feeder cells and sterile conditions, as the cells need to be cultured for 1-2 weeks.

c) It is safe and non-radioactive. In the LDA, however, the cultured cells are assayed in a cytotoxic T cell assay (CTL) assay using the radioactive isotope chromium-51.

d) It is an immediate *ex vivo* assay. As such it measures effector cells in their natural state without the introduction of unknown biases that occur as cells proliferate *in vitro* culture with antigen and exogenous cytokines.

e) The assay is performed over only 6 hours; as such it measures peptide-specific effector cells directly, without requiring these cells to proliferate *in vitro*. The short duration of the assay also eliminates the possibility that the cells may be becoming activated *in vitro*; it therefore measures effector function that is present *in vivo*. LDAs require the cells to proliferate many fold; however many effector cells do not proliferate in these conditions and therefore the result of the LDA is often an underestimate of the true number of circulating effectors.

The assay technique is expected to be of value in a number of different ways:-

i) For research into the mechanisms involved in peptide presentation and recognition and activation. Through the experimental work described in outline below, the inventors have gained insight into the phenotype and effector function of antigen-specific T-cells freshly isolated from peripheral blood.

ii) For quantifying peptide-specific effectors in HIV-infected individuals direct from peripheral blood.

iii) For monitoring the progress of, or resistance to, a chronic

backed microtitre plates. Input cell numbers were usually 5 x10^5 per well, and all assays were done in duplicate wells. Peptides were usually added to a final concentration of 1-2 μm, except in one peptide titration experiment where the concentration of the M1 58-66 peptide was diluted to 20 nM. Assays were usually performed for 12-14 hours, but certain assays were performed for 6 hours to confirm that the antigen-specific cells were capable of immediate effector function. Incubation was carried out at 37°C in an atmosphere containing 5% CO_2. Incubation was arrested by shaking off the contents of the wells and washing. Then 100 μl of 1 μg/ml of a biotinylated second anti-IFN-γ MAB 7-B6-1-biotin (Mabtech, Stockholm, Sweden) was added to the wells and the plates incubated for 3 hours. 100 μl of 1:1000 dilution of streptavidin-alkaline phosphatase conjugate was added to the wells and the plates incubated at room temperature for a further 2 hours. The wells were again washed, and 100 μl of chromogenic alkaline phosphatase substrate diluted 1:25 with deionised water was added to the wells. After a further 30 - 60 minutes incubation at room temperature the wells were washed to terminate the colorimetric reaction. The spots were counted under x20 magnification with a dissecting stereomicroscope.

Results

Using 5 well-defined HLA Class I-restricted influenza epitopes, free peptide was added to a final concentration of 2 μm directly to the freshly isolated PBMC in the ELISPOT assay. For almost all the individuals tested in this way, using epitopes restricted by HLA Class I alleles present in the individual, IFN-γ secreting peptide-specific effector T-cells were detected. Table 1 summarises the responses to these five epitopes. Most of these assays were performed over 12 - 14 hours, and Figure 1 is a bar chart showing the response at different concentrations of PBMC per well. However, to exclude the possibility that memory T-cells

number to the input number of fresh PBMC gives a measure of the relative frequency of circulating activated peptide-specific CD8+ effectors in peripheral blood. The frequency of IFN-γ-secreting CD8+ effectors for the HLA-A2.1-restricted epitope M158-56 in donor WB was measured by the invention assay (1/15000) and by conventional limiting dilution analysis (LDA) (1/103000).

Table 1. Class I-restricted influenza epitopes recognised by freshly isolated CD8+ effector T cells

Protein	Sequence	MHC class I restriction	No of Responders	No of donors tested
M1 58-66	GILGFVFTL	A2.01	6	6
NP 380-388	ELRSRYWAI	B8	3	4
M1 128-135	ASCMGLIY	B35	2	2
NP 265-273	ILRGSVAHK	A3	1	2
NP 383-391	SRYWAIRTR	B27.05	1	1

EXAMPLE 2

Application to quantifying peptide-specific effectors in HIV-infected individuals direct from peripheral blood

Cryopreserved peripheral blood lymphocytes (PBL) that had been freshly isolated from the peripheral blood of patient 868 were plated out at 50,000 cells per well of a PVDF coated 96 well plate, which had previously been coated with anti-interferon-gamma monoclonal antibody. Duplicate wells were set up for each antigen. Two types of duplicate

CLAIMS

1. A method of assaying for peptide-specific T-cells, which method comprises providing a fluid containing fresh cells in contact with a surface carrying an immobilised first antibody to a cytokine, adding a peptide to the fluid, incubating the resulting fluid mixture under conditions to cause any peptide-specific T-cells that have been pre-sensitised to the peptide to secrete the cytokine, and detecting any secreted cytokine bound to the immobilised first antibody.

2. A method as claimed in claim 1, wherein the fresh cells are peripheral blood mononuclear cells.

3. A method as claimed in claim 1 or claim 2, wherein the peptide-specific T-cells are CD8+ or CD4+ cells and the cytokine is IFN-γ .

4. A method as claimed in any one of claims 1 to 3, wherein the peptide is 7 - 12 amino acid residues in length.

5. A method as claimed in any one of claims 1 to 4, wherein the resulting fluid mixture is incubated under non-sterile conditions.

6. A method as claimed in any one of claims 1 to 5, wherein a second antibody to the cytokine labelled with a detectable moiety is used to detect any secreted cytokine bound to the immobilised first antibody.

7. A method as claimed in any one of claims 1 to 6, wherein the peptide is a known epitope.

8. A method as claimed in any one of claims 1 to 7, wherein the fresh cells are taken from a patient known to be suffering, or to have suffered from, infection with an intracellular pathogen.

9. A method as claimed in any one of claims 1 to 8, performed to monitor progress of HIV infection.

Figure 1

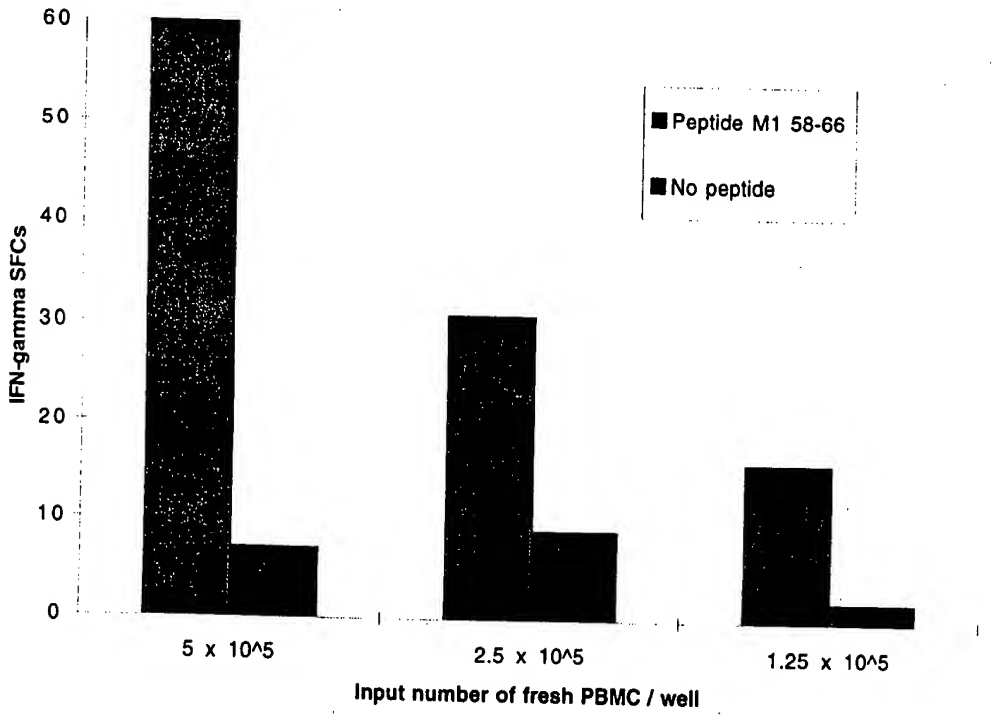
60
50
40
30
20
10
0
IFN-gamma SFCs
Peptide M1 58-66
No peptide
5 x 10^5
2.5 x 10^5
1.25 x 10^5
Input number of fresh PBMC / well

Figure 2

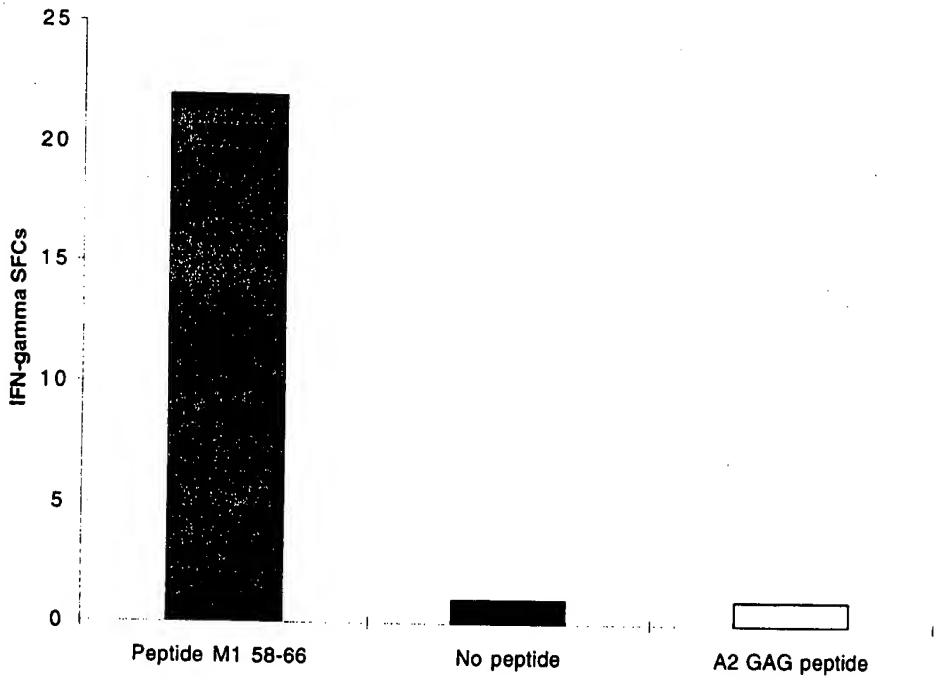
25
20
15
10
5
0
IFN-gamma SFCs
Peptide M1 58-66
No peptide
A2 GAG peptide

Figure 3

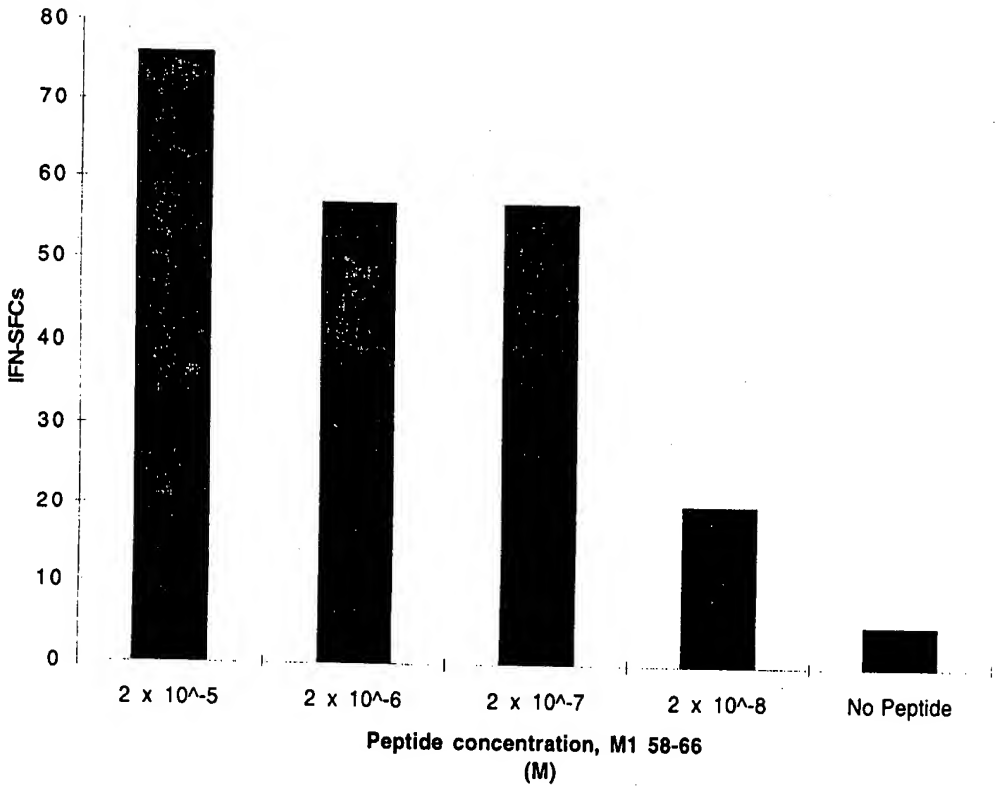
80
70
60
50
40
30
20
10
0
IFN-SFCs
2 x 10^-5
2 x 10^-6
2 x 10^-7
2 x 10^-8
No Peptide
Peptide concentration, M1 58-66
(M)

Figure 4

Enumeration of peptide-specific effectors from fresh Peripheral Blood Lymphocytes (PBL): comparison of several epitopes in an HIV infected patient (donor868)